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Dynamic expression of protective CEACAM1 on melanoma cells during specific immune attack

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Summary

An efficient immune response against tumours depends on a well-orchestrated function of integrated components, but is finally exerted by effector tumour-infiltrating lymphocytes (TIL). We have previously reported that homophilic CEACAM1 interactions inhibit the specific killing and interferon-γ (IFN-γ) release activities of natural killer cells and TIL. In this study a model for the investigation of melanoma cells surviving long co-incubation with antigen-specific TIL is reported. It is demonstrated that the surviving melanoma cells increase their surface CEACAM1 expression, which in turn confers enhanced resistance against fresh TIL. Furthermore, it is shown that this is an active process, driven by specific immune recognition and is at least partially mediated by lymphocyte-derived IFN-γ. Similar results were observed with antigen-restricted TIL, either autologous or allogeneic, as well as with natural killer cells. The enhanced CEACAM1 expression depends, however, on the presence of IFN-γ and sharply drops within 48 hr. This may be a mechanism that allows tumour cells to transiently develop a more resistant phenotype upon recognition by specific lymphocytes. Therefore, this work substantiates the melanoma-promoting role of CEACAM1 and marks it as an attractive target for novel immunotherapeutic interventions.

Keywords: cell surface molecules; T cells; tolerance/suppression/anergy; tumour immunity

Introduction

The interaction between the immune system and tumours is complex, especially because it may be persistent. This can lead to superimposed effects of selection, cell alterations and acquisition of new attributes that affect the total balance. Delineation of the subsequent effects of persistent tumour–host interactions that favour tumour growth is crucial for the development of novel anti-cancer regimens.

CEACAM1 is a transmembrane glycoprotein that belongs to the carcinoembryonic antigen family (part of the immunoglobulin superfamily), which encompasses several forms of proteins with different biochemical properties, all encoded on chromosome 19.[1] The CEACAM proteins share a common basic structure of sequentially ordered, different immunoglobulin-like domain(s). They may appear in two forms of cytosolic tail, a long form containing immunodominant tyrosine-based inhibitory motif (ITIM) and a short form devoid of ITIM.[2] These proteins are able to interact with each other.[2] For example,

CEACAM1 interacts homophilically with CEACAM1[3,4] and heterophilically with CEACAM5 but not with CEACAM6.[5] CEACAM1 is broadly distributed on a variety of cells, e.g. epithelial cells, melanoma and immune cells.[1] Many different functions have been attributed to the CEACAM1 protein. The CEACAM1 protein exhibits anti-proliferative properties in carcinomas of the colon[6] and prostate.[7] Additional data support the central involvement of CEACAM1 in angiogenesis,[8] metastasis[9] and insulin clearance.[10] CEACAM1 also has a role in the modulation of innate and adaptive immune responses because it is expressed on some lymphocyte subpopulations, such as CD16$^-$ natural killer (NK)[11,12] and activated T cells.[12] T-cell inhibition through engagement of CEACAM1 has been demonstrated by direct T-cell receptor (TCR) cross-linking[13] and via binding of *Neisseria* opacity associated (opa) proteins.[14] The inhibitory effect of CEACAM1 is exerted by the recruitment of Src homology domain 2-containing tyrosine phosphatase-1 (SHP-1) to the cytosolic ITIM sequences.[15] Lymphocytes express only the

CEACAM1 isoform that bears a long cytosolic tail.[11] We have previously shown that the CEACAM1 homophilic interactions[16–18] and the CEACAM1 heterophilic interactions with CEACAM5[19] inhibit NK-mediated killing independently of major histocompatibility complex (MHC) class I recognition. CEACAM1-mediated inhibition of NK cell function was dependent on the cytosolic tail.[16] CEACAM1 homophilic interactions seem important in some cases of metastatic melanoma because increased CEACAM1 expression on NK cells derived from patients has been observed compared with cells from healthy donors.[16] Indeed, a strong association has been reported between CEACAM1 expression on primary cutaneous melanoma lesions[9] or on primary non-small cell lung cancer[20,21] and the development of metastatic disease and poor survival.

Attention to treatments based on adoptive cell transfer (ACT) of tumour-infiltrating lymphocytes (TIL) has increased, especially in view of a recent clinical trial of TIL ACT treatment in 35 patients with refractory metastatic melanoma. In this specific study, up to 50% of the patients exhibited a significant clinical response, with some of the patients remarkably achieving a complete response.[22] However, although these results are encouraging, TIL-based treatment is still far from its full potential. We have recently demonstrated that CEACAM1 expression on TIL increases following clinical ACT expansion protocols for refractory melanoma patients.[23] The combination of CEACAM1 inhibitory effect on TIL,[23] increased CEACAM1 expression during expansion protocols[23] and common CEACAM1 expression on melanoma[16,23] suggests that CEACAM1 homophilic interactions may hinder TIL function *in vivo* and hence diminish the efficacy of TIL ACT treatments. We have previously reported a preliminary observation that melanoma cells surviving long coculture *in vitro* with specific TIL express higher levels of CEACAM1, while no similar change could be observed on the surface of the attacking TIL.[23]

The objective of this study is to characterize the dynamic expression of CEACAM1 by melanoma cells during an ongoing attack by tumour-reactive lymphocytes *in vitro* and to investigate the underlying mechanisms. We shall demonstrate that CEACAM1 up-regulation by surviving melanoma cells is an active process, driven by specific immune recognition and dependent on lymphocyte-derived interferon-γ (IFN-γ). Enhanced CEACAM1 expression protects the surviving melanoma cells against subsequent attack by fresh lymphocytes. However, it depends on the continuous presence of IFN-γ, because it drops sharply to basal levels following elimination of IFN-γ. Our laboratory findings therefore suggest that melanoma cells can actively develop a transient phenotype of enhanced resistance during lymphocyte-mediated attack by coupling CEACAM1 expression with responsiveness to IFN-γ.

Materials and methods

Cells and media

The TIL clones used in this study were: L2D8 [directed against the gp100-derived peptide 209–217 in complex with human leucocyte antigen (HLA) -A*02] and JKF6 (directed against the MART1-derived peptide 27–35 in complex with HLA-A*02). Human melanoma cell lines were the HLA-A2-positive 526mel (HLA-A*02, -A*03, -B*15, -Cw*03) and 624mel (HLA-A*02, -A*03), the HLA-A2-negative 938mel (HLA-A*0101, -A*2402, -B*0702, -B*0801, -Cw*0701, -Cw*0702) and LB33B1. In addition, the NK92 cell line [American Type Culture Collection (ATCC), Manassas, VA] and 721.221 Epstein–Barr virus-transformed B-cell lymphoma (ATCC) were used. Melanoma cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Minneapolis, MN) supplemented with: 10% heat inactivated fetal calf serum; 1 mM penicillin–streptomycin; 1 mM non-essential amino acids; 1 mM L-glutamine and 1 mM sodium pyruvate (all from Gibco). The NK92 cell line was grown as suggested by the ATCC. Complete RPMI-1640 (Gibco) medium included 10% heat inactivated fetal calf serum, 1 mM penicillin–streptomycin, 1 mM non-essential amino acids, 1 mM L-glutamine and 1 mM sodium pyruvate.

Primary melanoma and TIL bulk cultures

Primary melanoma cultures were developed from specimens as previously described,[24] and included the MEL05 cells (HLA-A*29, -B*1402, -B*3502, -Cw*04, -Cw*0802) and MEL09 cells (HLA-A*02, -A*26, -B*51, -B*38, -Cw*14, -Cw*12). Primary TIL bulk cultures were obtained from surgically excised melanoma specimens and included: TIL001, TIL01, TIL02, TIL05F1, TIL05F10 and TIL09. The TIL bulk cultures were grown as formerly reported.[24] TIL05F1 and TIL05F10 served as reactive TIL in assays against autologous MEL05 melanoma cells and TIL09 against autologous MEL09 cells. TIL001, TIL01 and TIL02 do not recognize any of the melanoma cells used in this study and have therefore been used as inert TIL controls for specificity. These TIL, however, can be stimulated with non-specific TCR stimulation (data not shown).

Antibodies

The following purified monoclonal antibodies (mAbs) were used: Kat4c mAb directed against human CEACAM1, -3, -5, -6, -8 (Dako, Glostrup, Denmark), NC8 mAb directed against human CEACAM1 [kind gift of Dr Miguel Lopez-Botet, Universitad Pompeu Fabra (DCEXS), Barcelona, Spain], rabbit anti-human polyclonal anti-CEACAM (Dako), control immunoglobulin G (IgG) (Serotec, Oxford, UK), pan anti-human MHC class I

mAb W6/32, anti-human CD99 mAb 12E7 and mAb anti-human IFN-γ (BD Pharmingen, San Diego, CA). The conjugated mAbs used in this work were: fluorescein isothiocyanate (FITC)-conjugated Kat4c (Dako), phycoerythrin (PE)-conjugated anti-MCSP (Miltenyi Biotec, Bergisch-Gladbach, Germany), allophycocyanin-conjugated anti-CD56 (R&D Systems, Minneapolis, MN) and biotin-conjugated anti human IFN-γ (Pharmingen). Secondary antibodies included FITC-conjugated goat anti-mouse IgG F(ab')$_2$ (ICN, Irvine, CA) and PE-conjugated goat anti human IgG (Jackson Immunoresearch, West Grove, PA).

Immunoglobulin fusion proteins

The CEACAM1–immunoglobulin fusion proteins (kind gift of Dr Ofer Mandelboim, Lautenberg Centre for General and Tumour Immunology, Hadassah Medical School, Jerusalem, Israel) were produced and utilized according to a previous report.[17] Briefly, the complementary DNA of the extracellular portion of each receptor was amplified by polymerase chain reaction and cloned in frame into a vector containing the Fc portion of human immunoglobulin. Constructs were then transfected into COS-7 cells. The immunoglobulin fusion proteins were purified on a protein G column, divided into aliquots, stored at −20° and routinely analysed for degradation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Long coculture assays and treatment with recombinant IFN-γ

Long coculture assays were performed in 48-well plates. Twenty thousand melanoma cells were allowed to adhere firmly to the well for 4 hr in complete RPMI-1640. Media were removed and 50 000 TIL, clones or bulk cultures, were added in 600 μl complete medium per well. Plates were cultured in a humidified 5% CO$_2$ incubator for 4 days. Monitoring of cells and supernatants was conducted daily; therefore, multiple well replicates for each time-point were prepared in advance at day 0. Once a day, culture medium containing both supernatants and floating cells was collected from all designated well replicates and centrifuged. The IFN-γ was quantified in the supernatants and cell phenotype was determined by fluorescence-acitvated cell sorting (FACS). The remaining adherent cells were gently washed with phosphate-buffered saline, trypsinized and analysed by FACS. Adherent cells were comprised chiefly of viable melanoma cells, with less than 10% non-viable cells, and were therefore considered as 'surviving cells'. Floating cells, on the other hand, were comprised chiefly of viable TIL and some melanoma cells that were mostly propidium iodide (PI)-positive. Surviving melanoma cells could be easily differentiated from residual TIL in flow cytometry based

on forward scatter (FSC) over side scatter (SSC) parameters, as verified by staining for the melanoma-specific antigen MCSP and CD3.[23] The presence of various markers on surviving cells was expressed as the 'Ratio' of staining median fluorescence intensity (MFI) on surviving cells divided by the MFI of the same staining on the same cells cultured in the absence of TIL. In conditioned medium experiments, the expression ratio of various molecules was calculated by dividing the MFI of cells incubated in conditioned medium or control conditioned medium by cells incubated in regular culture medium. Neutralizing anti-IFN-γ mAb was used in a final concentration of 10μg/ml. The concentration of recombinant IFN-γ (Chemicon International, Temecula, CA) included in the culture did not exceed 50 ng/ml.

Cytotoxicity assays and quantification of IFN-γ secretion

Cytotoxicity assays were performed by PI costaining of carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labelled target cells; 5×10^6 target cells were labelled with 2·5 μM CFSE, which strongly labels cells and allows differentiation between target and effector cells. Ten thousand labelled target cells were coincubated in 96 U-shaped microplates with given amounts of effector cells for 5 hr in a humidified 5% CO$_2$ incubator. Killing rate was assessed by the percentage of PI costaining cells out of gated CFSE-labelled cells. In all experiments the percentage of PI-positive CFSE-labelled cells in wells cultured without effectors did not exceed 15%. The amount of IFN-γ in supernatants was evaluated by standardized enzyme-linked immunosorbent assay (R&D Systems).

Generation of CEACAM1-silenced melanoma cells

Generation of CEACAM1-silenced melanoma cells was carried out using commercially available small interfering RNA (siRNA) target sequences cloned in the MISSIOM™ short hairpin RNA system (lentiviral plasmids pLKO.1-puro) (Sigma-Aldrich, Rehovot, Israel) and lentiviral expression system. Viral particles were produced by transient liposome-mediated cotransfection (FuGENE6, Roche, Indianapolis, IN) of 293T cells with the vectors pCMVΔ8.9, pVSVG and each vector containing the designated siRNA sequence (Sigma-Aldrich). Culture medium was replaced after 24 hr with fresh complete RPMI-1640 medium, and incubated for an additional 24 hr. The medium containing viral particles was collected and filtered through a 0·45-μm syringe filter. Infection was carried out using the filtered medium along with 1 μg/ml polybren on melanoma cells. Virus-containing medium was removed after 6 hr and replaced with fresh medium. Double infections were performed by generation of single-clone viral particles in a concentrated volume, and introduced together onto the target

cells. This procedure was performed twice, on sequential days. Selection medium of 0·5 μg/ml puromycin, in complete RPMI-1640, was introduced 3 days after infection. Cells were grown in selection medium for at least 2 weeks, before being used for experiments. The sequence of the scrambled control siRNA was not specified by the manufacturer. The following are the names of the clones and their respective accession numbers as specified by the manufacturer (Sigma-Aldrich): Clone ID: NM_001712.2-881s1c1 Accession Number(s): NM_001024912.1, NM_001712.3; Clone ID: NM_001712.2-757s1c1 Accession Number(s): NM_001024912.1, NM_001712.3; and Clone ID: NM_001712.2-1248s1c1 Accession Number(s): NM_001024912.1, NM_001712.3. The DI #1 cells were generated by transfection with the sequences 881 and 757. The DI #2 cells were generated by transfection with the sequences 881 and 1248. The control RNA cells were generated by transfecting twice with the scrambled sequence.

Results

Melanoma cells surviving TIL-mediated attack increase the expression of CEACAM1 and MHC class I but not CD99

We have previously reported that CEACAM1 expression is up-regulated on 526mel melanoma cells surviving attack by a MART1-specific TIL clone (JKF6), while it remains unaltered on the JKF6 cells.[23] To further investigate this phenomenon we expanded the experimental setup to include melanoma cell lines (HLA-A2-positive 526mel cells and HLA-A2-negative 938mel cells) and TIL clones (HLA-A2-restricted L2D8 or JKF6 clones and HLA-mismatched TIL001, all from allogeneic sources), as well as primary melanoma (MEL05) and TIL cognate cultures (TIL05F1 or TIL05F10). Analysis included MHC class I and CD99 in addition to CEACAM1. Supernatants, floating cells and adherent cells were collected daily from designated wells for analysis. The experimental methodology is detailed in the *Materials and methods* section.

A three-fold increase in the CEACAM1 expression ratio (from baseline MFI of 100 to around 300) was observed on the surface of 526mel cells surviving specific attack by reactive L2D8 or JKF6 TIL clones on day 3 (Fig. 1a). In another study that used renal cell carcinoma with allogeneic CD8 cells a similar observation was reported.[25] No similar phenomenon could be observed when the 526mel cells were coincubated with the inert TIL001 cells (Fig. 1a). Therefore, specific recognition is probably required, and the possibilities of metabolic stress as a result of the mere presence of TIL or of independent non-TCR-mediated interactions are not favoured as alternative explanations. Supporting this suggestion, when the HLA-A2-negative 938mel cells (MFI of CEACAM1 expression baseline = 35), which are not recognized by the L2D8, JKF6 or TIL001 cells, were used, the CEACAM1 expression ratio did not change (Fig. 1b). Importantly, a two-fold enhancement in the CEACAM1 expression ratio (from a baseline MFI of 45 to around 90) was also observed in primary MEL05 cells surviving specific attack by the cognate reactive TIL05F1 or TIL05F10 cells, but not with the inert TIL001 (Fig. 1c). It is still impossible to conclude at this stage whether this occurrence represents the selection of melanoma cells with stronger CEACAM1 expression or whether the expression pattern of CEACAM1 is actively altered by the surviving cells.

A similar pattern of increased expression ratio on melanoma cells surviving specific TIL-mediated attack was observed with MHC class I. The ratio of MHC class I expression was increased by up to seven-fold with similar kinetics over time on the surface 526mel cells incubated with reactive L2D8 or JKF6 cells but not with inert TIL001 cells (Fig. 1d). No similar phenomenon could be documented when the HLA-A2-negative 938mel cells were used (Fig. 1e). MHC class I up-regulation was observed with primary MEL05 cells surviving specific attack by the autologous TIL05F1 or TIL05F10 cells (Fig. 1f). The increased expression ratios of CEACAM1 and MHC class I on surviving melanoma cells did not seem to be incidental because no significant change in the expression ratio of CD99 could be seen in any of the settings (Figs 1g–i). These observations suggest that this process is driven by the specific recognition of melanoma cells by TIL. Similar results were observed with similar kinetics with another melanoma cell line and reactive or inert TIL (data not shown).

There is no correlation between TIL killing activity and CEACAM1 enhancement by surviving melanoma cells

Two main explanations may account for this immune recognition-dependent phenomenon: (1) selection of specific melanoma subpopulations that express higher levels of CEACAM1, (2) active up-regulation. During the long coincubation setup described above, the killing and IFN-γ secretion activities of both L2D8 and JKF6 were tested daily. Both TIL clones exhibited specific secretion of IFN-γ in the presence of 526mel but not 938mel (Fig. 2a,c). Surprisingly, however, a considerable difference in the killing activity was observed, as L2D8 displayed only moderate cytotoxic activity around 10%, while JKF6 demonstrated strong cytotoxic activity of more than 30% (Fig. 2b and d). Importantly, there were no significant differences in the enhancement patterns of CEACAM1 or MHC class I on 526mel cells surviving either one of the TIL clones (Fig. 1a,d). The specificity of L2D8 and JKF6 was verified because minor or no killing or secretion of IFN-γ was observed in the presence of 938mel cells (Fig. 2a–d). This could imply that killing activity may have less significance
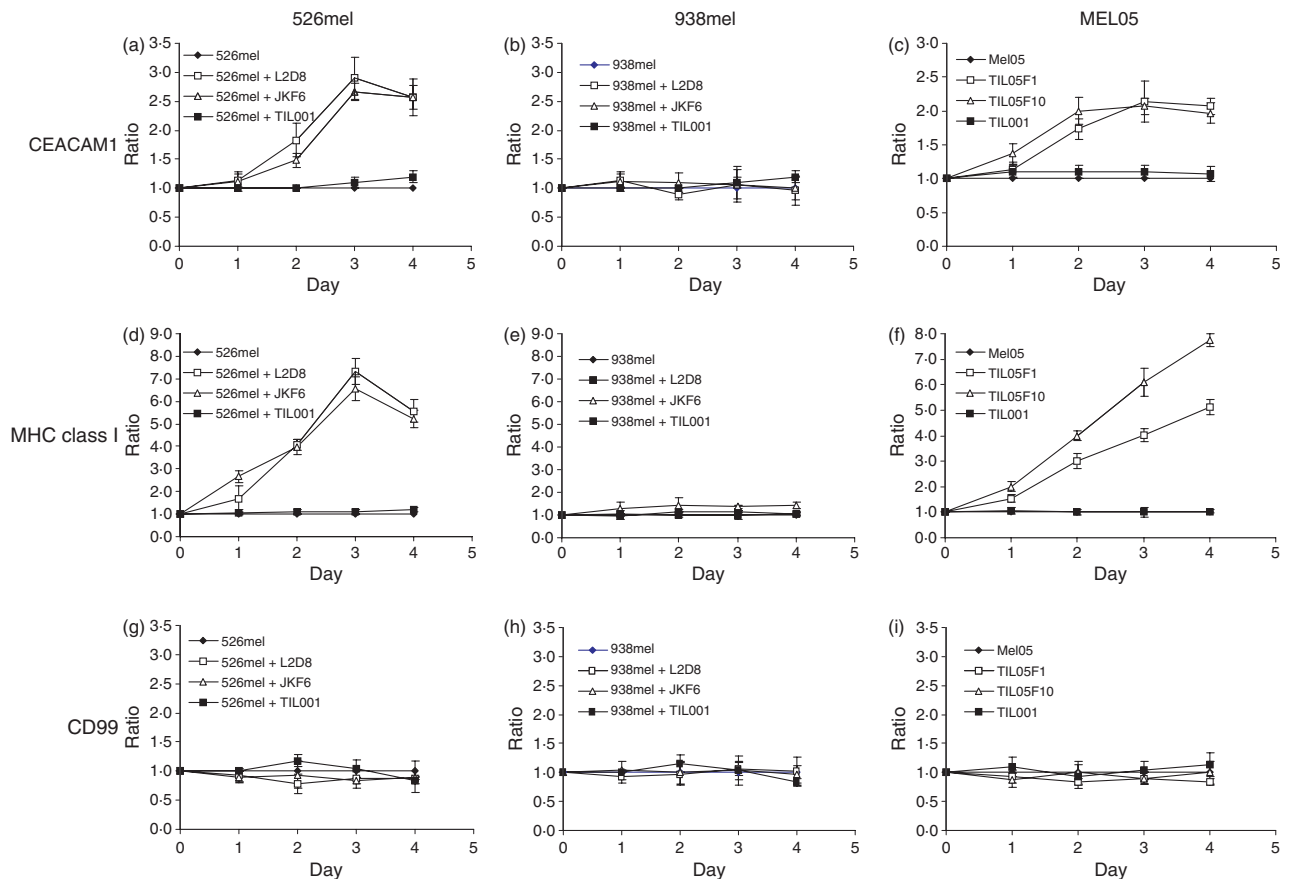
Figure 1. CEACAM1 expression is increased on melanoma cells surviving specific tumour-infiltrating lymphocyte (TIL)-mediated attack. Melanoma cells lines 526mel and 938mel were cocultured for 4 days either with reactive JKF6 or L2D8 TIL clones or with inert TIL001 cells. Primary MEL05 cells were cocultured for 4 days either with reactive TIL05F1 or TIL05F10 or with inert TIL001 cells. Every day, the surviving melanoma cells were isolated and analysed by flow cytometry for expression CEACAM1 (a–c), major histocompatibility complex (MHC) class I (d–f) or CD99 (g–i). Discrimination of melanoma cells from remnants of TIL was verified by costaining for MCSP (MCSP-positive cells). The y-axis represents the ratio of expression, which was calculated daily as [mean fluorescence intensity (MFI) of a given staining on surviving melanoma cells] / (MFI with the same monoclonal antibody on melanoma cells cultured without TIL). Background was a ratio of 1. The figure shows the mean results of five independent experiments.

in causing up-regulation of CEACAM1 and MHC class I on surviving 526mel cells.

Therefore similar experiments were conducted with another primary TIL bulk culture (TIL09) against autologous MEL09 cells. In this cognate pair, a prominent killing activity could be seen (Fig. 2f), but no IFN-γ could be detected (Fig. 2e). Specificity of TIL09 was verified against HLA-mismatched 526mel cells because no effector functions were identified (Fig. 2e,f). In this case, however, despite the efficient recognition and killing activity exhibited by TIL09, no change either in the pattern of CEACAM1 or MHC class I could be revealed among the surviving MEL09 cells (Fig. 2g). Similar results were obtained with additional bulk cultures (data not shown). These results show that immune recognition of the melanoma cells was mandatory for enhancement of CEACAM1 and MHC class I on surviving cells (Figs 1 and 2). However, there was no correlation between killing activity

and this occurrence (Fig. 2b and d; Fig. 1a and d; Fig. 2e–g). It seems that under these conditions a simple selection process of melanoma subpopulations with higher CEACAM1 expression by killing does not play a significant role. Nevertheless, it implies that effector functions other than cytotoxicity may play a role in enhancing CEACAM1 and MHC class I on surviving melanoma cells. Indeed, this was observed only when IFN-γ was secreted, as shown by L2D8 (Fig. 2a) and JKF6 (Fig. 2c), but not by TIL09 (Fig. 2e).

Conditioned medium causes up-regulation of CEACAM1 on melanoma cells

Since enhancement of CEACAM1 depends on immune recognition (Fig. 1) and is probably independent of killing activity (Fig. 2), we hypothesized that secreted proteins might induce CEACAM1 expression. Therefore,
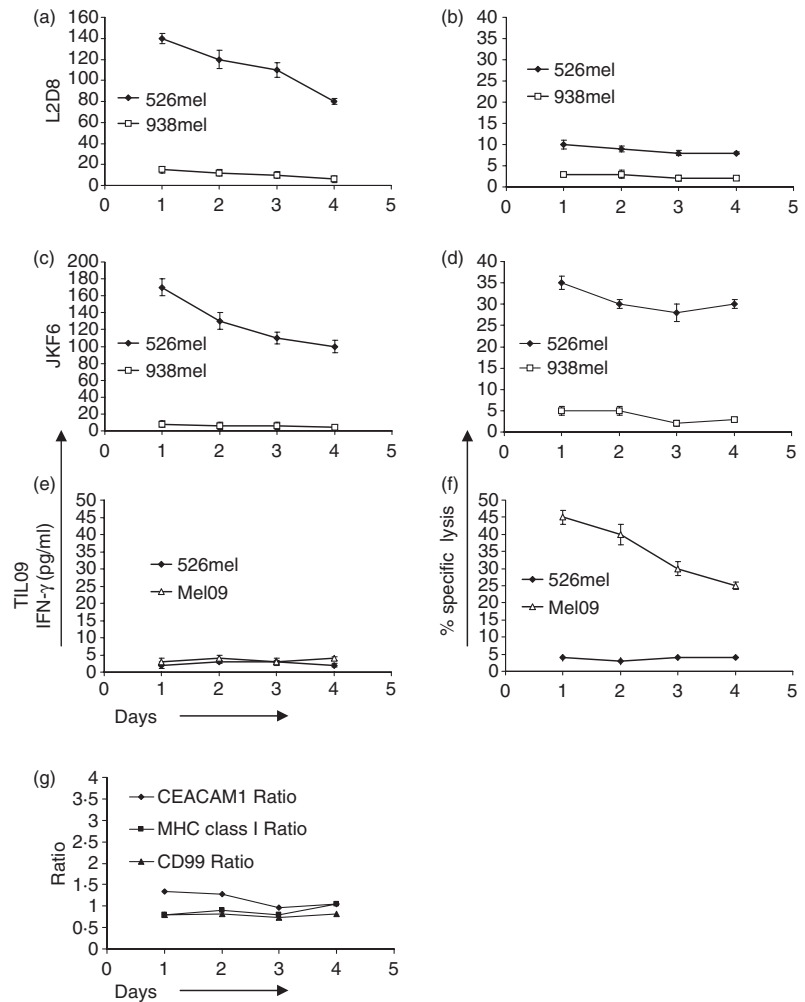
Figure 2. Enhanced CEACAM1 expression correlates with cytokine secretion but not with killing activity. Various tumour-infiltrating lymphocytes (TIL; indicated in the left) were cocultured either with cognate melanoma cells or with irrelevant melanoma cells for 4 days. The amount of interferon-γ (IFN-γ) secreted into the medium (a,c,e) and TIL killing activity (b,d,f) were monitored daily, as well as the expression ratios of CEACAM1, major histocompatibility complex (MHC) class I and CD99 on surviving MEL09 melanoma cells (g). The expression profile on surviving 526mel cells is described in Fig. 1(a,d,g). Background is a ratio of 1. The figure shows one representative experiment out of five performed.

conditioned media were collected on the first or second days of long coculture experiments of either reactive JKF6 cells (CM) or inert TIL001 cells (Control CM) with 526mel cells. Fresh 526mel cells were cultured for 4 days either with fresh medium, CM or Control CM. Melanoma cells were stained daily for the expression of CEACAM1, MHC class I and CD99, as well as for CEACAM1-binding capacity with the CEACAM1–immunoglobulin fusion protein. Strikingly, the staining ratios of CEACAM1, CEACAM1–immunoglobulin and of MHC class I on 526mel cells cultured in CM increased over time, reaching a maximum on day 3 (Fig. 3a–c). The Control CM had little or no effect (Fig. 3a–c). Importantly, the CD99 ratio did not change significantly, attesting to the specificity of CEACAM1 and MHC class I up-regulation (Fig. 3d). Similar results were gathered with CM generated via the coincubation of other reactive TIL, such as TIL05F1 or TIL05F10 with the cognate MEL05 cells, but not of TIL09 with MEL09 (data not shown). This demonstrates that a soluble factor derived from lymphocytes following specific target recognition induces CEACAM1 and MHC class I expression, but not CD99.

Indeed, dilution of CM with Control CM abolished the induction of CEACAM1 and MHC class I expression. The induction of CEACAM1 was completely eliminated when CM was diluted to 20% whereas some increased expression ratio of MHC class I was still evident even when CM was diluted to 3% (data not shown). Incubation of TIL or clones in the presence of CM did not change CEACAM1 expression (data not shown) nor did it alter TIL cytotoxic or IFN-γ secretion activities (data not shown).

IFN-γ is responsible for up-regulation of CEACAM1 on surviving melanoma cells

The findings described above, including the coupling of CEACAM1 induction with MHC class I (Fig. 1), which is up-regulated by IFN-γ,[26] suggest that this process is triggered by IFN-γ. Indeed, when a neutralizing anti-IFN-γ mAb was added to the CM, both induction of CEACAM1 expression and binding capacity were considerably impaired (Fig. 3a,c – grey bars). Induction of MHC class I was impaired as well, but to a lesser extent (Fig. 3b). The expression of CD99 did not change in the presence
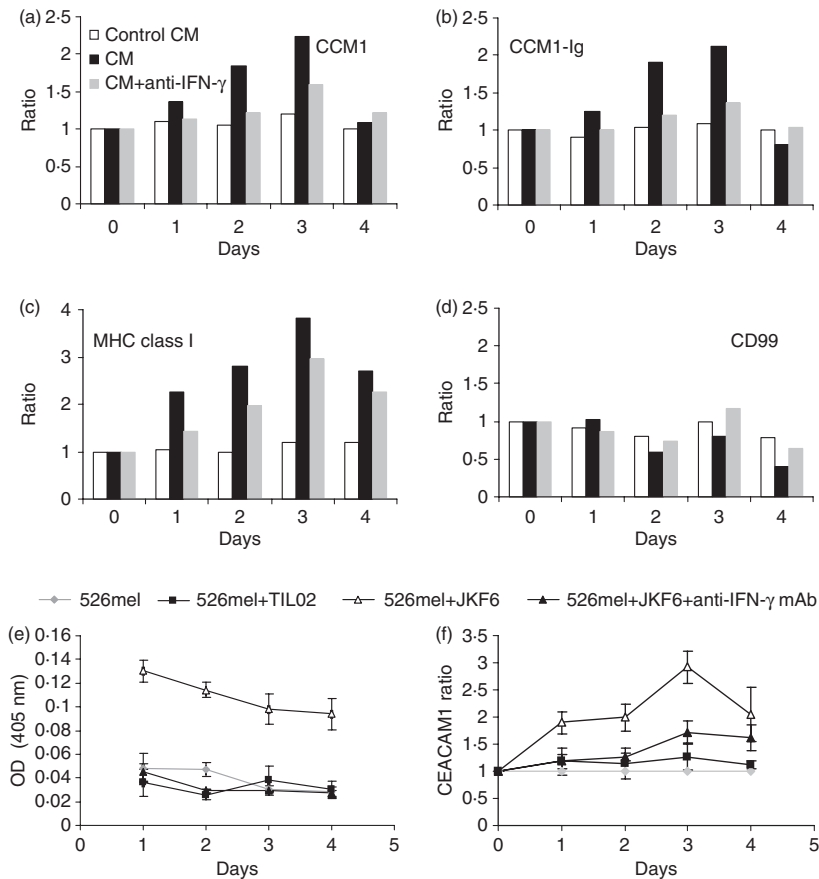
Figure 3. Interferon-γ (IFN-γ) is responsible for the up-regulation of CEACAM1 on melanoma cells. 526mel cells were incubated only with conditioned medium of 526mel incubated with inert TIL001 cells (Control CM – white bars), or of 526mel incubated with JKF6 cells with or without neutralizing anti-IFN-γ monoclonal antibody (CM alone – black bars or CM plus anti-IFN-γ – grey bars). The expression of various molecules was tested, as indicated in each graph (a–c). (d) The CEACAM1 binding capacity to CEACAM1-Ig. The *y*-axis shows the expression ratio as detailed in the *Materials and methods* section. The figure shows one representative experiment out of four performed. (e–f) A prolonged coincubation of 526mel cells either alone or with inert TIL02 cells, or with reactive JKF6 cells in the presence or absence of neutralizing anti-IFN-γ monoclonal antibody, was performed. (e) The neutralization of IFN-γ in the culture medium as measured by enzyme-linked immunosorbent assay and (f) shows the abrogation of enhanced CEACAM1 expression ratio on surviving cells. Error bars represent deviation within the experiment. The figure shows one representative experiment out of three performed.

of CM with or without anti-IFN-γ (Fig. 3d). These results show that IFN-γ is responsible for up-regulation of CEACAM1 and concur with previous reports regarding the regulation of CEACAM1 expression by IFN-γ.[25,27]

Next, we neutralized IFN-γ during the long coculture assays. 526mel cells were incubated either alone, with inert TIL02 cells or with reactive JKF6 cells in the presence or absence of anti-IFN-γ mAb. Daily analysis of supernatants revealed the specific secretion of IFN-γ from reactive JKF6 cells, which could not be detected when the inert TIL02 cells were used (Fig. 3e). Notably, no IFN-γ could be detected in the supernatants of the 526mel cells cocultured with JKF6 cells in the presence of anti-IFN-γ mAb (Fig. 3e). A striking eradication of the up-regulation of CEACAM1 on 526mel cells surviving JKF6 was observed when anti-IFN-γ mAb was included in the assay (Fig. 3f). These results provide evidence that the specific secretion of IFN-γ from TIL is responsible for this phenomenon.

Further, addition of purified recombinant human IFN-γ to various melanoma cell lines, such as 526mel and 624mel, resulted in increased CEACAM1, but not CD99, expression even in the absence of TIL (Fig. 4a,c). The MHC class I up-regulation was observed as expected (Fig. 4b). Purified recombinant human IFN-γ was also

added to isolated primary TIL cultures before rapid expansion (TIL05F1 pre-REP) or after rapid expansion (TIL05F1 post-REP). Surprisingly, however, neither CEACAM1 nor MHC class I was induced even in the presence of high IFN-γ concentrations of 50 ng/ml (Fig. 4a–c).

CEACAM1 is up-regulated on melanoma cells following natural killer-mediated attack

Natural killer (NK) cells are a major source of IFN-γ, which is secreted upon interaction with tumours and virus-infected cells.[28] We utilized the NK92 cell line, which has intact cytotoxic and IFN-γ-release activities, in long coculture assays. LB33melB1 cells, which display some degree of susceptibility to NK cells,[29] were used as target cells. Long coculture assays were performed as described above. The surviving adherent melanoma cells were stained daily either for CEACAM1, MHC class I or CD99 with concomitant costaining for CD56 to allow differentiation of residual NK92 cells (CD56-positive) from the surviving melanoma cells (CD56-negative, data not shown). The ratio of expression was calculated as described above. Remarkably, both expression ratios of CEACAM1 and MHC class I were increased on the surface of the LB33melB1 (Fig. 5a). There was no significant
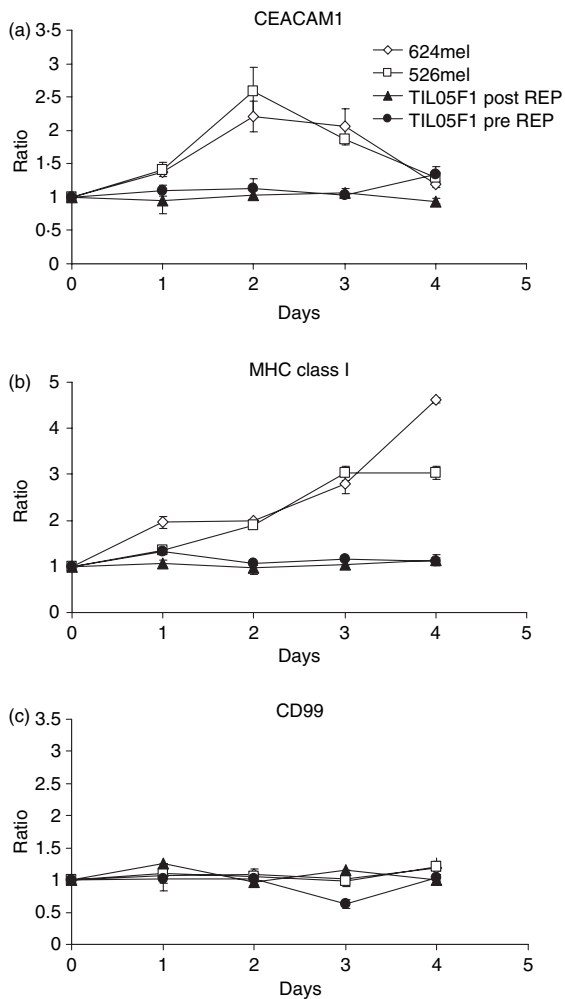
Figure 4. Recombinant interferon-γ (IFN-γ) induces functional CEACAM1 up-regulation on melanoma cells but not on tumour-infiltrating lymphocytes (TIL). Various melanoma cell lines (526mel, 624mel) or TIL, pre- or post-rapid expansion protocol, were incubated for 4 days in the presence of 50 ng/ml recombinant IFN-γ. The expression analysis of various molecules was tested over time, as indicated above each graph. The y-axis shows the expression ratio as detailed in the *Materials and methods* section. The figure shows one representative experiment out of four performed.

change in the ratio of CD99 (Fig. 5a). Furthermore, conditioned medium from NK cells coincubated with non-melanoma cells such as 721.221 B-lymphoma cells induced similar CEACAM1 expression on melanoma cell lines such as 526mel (Fig. 5b), 938mel and MEL05 (data not shown). Experiments with conditioned media and neutralizing anti-IFN-γ mAb, similar to those described above, have yielded similar results, demonstrating that this occurrence is also attributed to NK-derived IFN-γ (Fig. 5b). Specific recognition of tumour cells by any effector lymphocyte with the capability to secret IFN-γ may therefore result in up-regulation of CEACAM1 on the surviving cells.

Enhanced CEACAM1 expression on surviving melanoma cells depends on the continuous presence of IFN-γ

To test the persistence of enhanced CEACAM1 expression, 526mel melanoma cells were cocultured for 4 days either with JKF6, inert TIL001 or without TIL. The surviving melanoma cells were isolated after the fourth day, washed thoroughly and re-seeded in new culture plates either in fresh medium, conditioned medium of JKF6 and 526mel (CM) or conditioned medium of TIL001 and 526mel (Control CM). After an additional 4 days, half of the medium was carefully replaced with fresh medium of the same type (Fig. 6a, experimental design). Cells were analysed daily for expression of CEACAM1 and MHC class I during the first 4 days of coculture as well as during the additional 9 days after re-seeding (Fig. 6b,c). The basal expression level of CEACAM1 and MHC class I was determined before the coculture. As expected, a significant up-regulation in the expression of both CEACAM1 and MHC class I was observed during coculture only with JKF6 (Fig. 6b,c). The enhanced CEACAM1 expression documented on melanoma cells that survived coculture with JKF6 declined by 70% within 2 days after re-dispersion and incubation in fresh medium or Control CM, and returned to baseline over an additional 2 days (Fig. 6b). Enhanced CEACAM1 expression persisted only in CM (Fig. 6b). In contrast, high levels of MHC class I were maintained for 9 days in fresh medium or Control CM, although a gradual decrease could be observed starting from day 8 (Fig. 6c). Enhanced MHC class I expression continued in the presence of CM and remained high after day 8 as well (Fig. 6c). No significant changes could be observed in CEACAM1 or MHC class I expression levels in control cells over time (Fig. 6b,c). CM induced expression of CEACAM1 and MHC class I on melanoma cells that were not pre-exposed to JKF6 (data not shown). Monitoring of cell density revealed that all cells exposed to either JKF6 cells or to CM were growth inhibited but viable (data not shown). This concurs with the already described attributes of IFN-γ on cell cycle arrest.[30,31]

A similar experimental design was employed with recombinant IFN-γ. Melanoma cells were incubated in culture medium with or without recombinant IFN-γ for 4 days. Afterwards, cells were washed thoroughly and re-seeded in new culture plates in the presence or absence of IFN-γ for a further 7 days. Half of the medium was replaced with fresh appropriate medium 4 days after re-seeding (Fig. 6d). An expected up-regulation in both CEACAM1 and MHC class I expression levels was documented (Fig. 6e,f). However, CEACAM1 expression rapidly declined after IFN-γ was washed, at a rate of a 70% decrease during the first 2 days and a return to basal levels after two more days (Fig. 6e). The declining rate of CEACAM1 was similar to the rate observed following
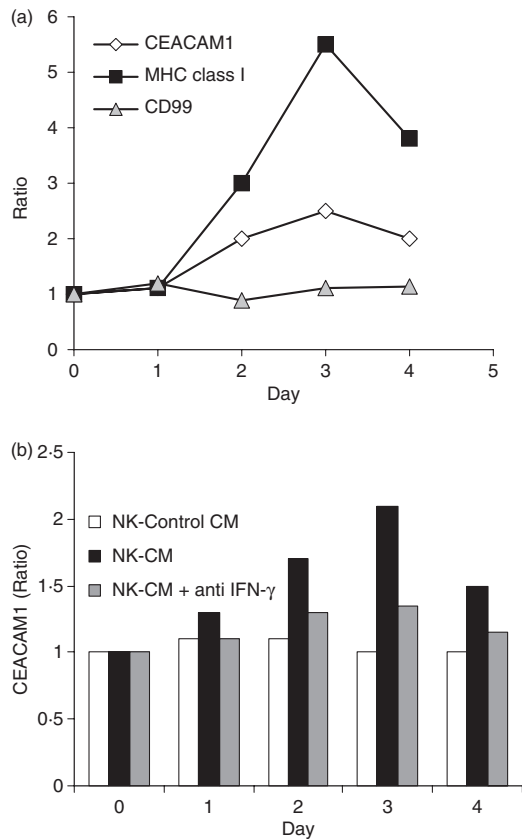
Figure 5. CEACAM1 expression is increased on melanoma cells that survive natural killer (NK)-mediated attack. (a) A prolonged coincubation of LB33mel.B1 with the NK92 cell line was performed. Discrimination of melanoma cells from remnants of NK92 cells was verified by costaining for CD56 (CD56-negative cells). The expression analysis of various molecules was tested over time, as indicated above each graph. The *y*-axis shows the expression ratio as detailed in the *Materials and methods* section. The figure shows one representative experiment out of three performed. (b) 526mel cells were incubated in the conditioned medium of NK92 and NK-sensitive 721.221 cells (NK-CM) with or without blocking anti-interferon-γ (IFN-γ) monoclonal antibody. The conditioned medium of NK92 and NK-resistant 938mel (NK-Control CM) was used as control. The *y*-axis shows the expression ration of CEACAM1 and the figure shows one representative experiment out of three performed.

washout of the CM (compare with Fig. 6b). In the continuing presence of IFN-γ, CEACAM1 expression was even further increased (Fig. 6e). In contrast, enhanced MHC class I expression was persistent for the first 2 days after removal of IFN-γ (Fig. 6f). Then, a slow decrease over the next 5 days was measured, but the expression level was still clearly above basal MHC class I levels (Fig. 6f). Concentration of IFN-γ in the medium did not exhibit a significant decrease over time (data not shown). Notably, a slight decrease in CEACAM1 and MHC class I expression in the presence of CM was observed on day 8, and was followed by an increase at day 9 (Fig. 6b,c). However, half of the medium was replaced at day 8 so

this observation is probably related to the general culture conditions.

These results show that enhanced CEACAM1 expression depends on the continuous presence of IFN-γ, as opposed to a more stable enhancement of MHC class I.

Increased CEACAM1 expression on surviving melanoma cells contributes to enhanced resistance to specific TIL-mediated killing

To assess the protective contribution of CEACAM1 to surviving melanoma cells, we generated 526mel cells that stably express siRNA targeted at CEACAM1, as described in the Materials and methods section. Several different target sequences have been selected for silencing of CEACAM1. Figure 7(a) shows CEACAM1 expression in two different representative clones (DI #1 and DI #2), each transfected with different target sequences, as compared to 526mel cells transfected with scrambled control sequence (Control-RNA).

Various 526mel transfectants were divided into two groups: (1) prelabelled with CFSE or (2) no pre-label. Each group was concomitantly coincubated with reactive JKF6 cells, inert TIL01-CN cells or in the absence of any TIL for 4 days. Reactivity of TIL was monitored by the detection of IFN-γ in the culture supernatant (data not shown). Surviving cells were obtained daily from all experimental setups. CEACAM1 and MHC class I expression were monitored as described above using the unlabelled cells. As observed in other experiments, both CEACAM1 and MHC class I expression increased only in the presence of reactive JKF6 cells (Fig. 7b,c). Importantly, although initial CEACAM1 expression in silenced 526mel cells was lower, the CEACAM1 up-regulation rate on surviving cells was similar to the rate observed with Control-RNA cells (Fig. 7b). Maximal CEACAM1 intensity on surviving DI #1 and DI #2 cells was around 50% of the Control-RNA cells (Fig. 7b). CEACAM1 silencing did not affect MHC class I expression or up-regulation on surviving cells, as compared to the Control-RNA cells (Fig. 7c). The presence of the inert TIL01-CN cells had little or no effect (Fig. 7b,c). There were no changes in the expression of CD99 (data not shown).

The surviving CFSE-labelled 526mel cells were isolated and tested for susceptibility to fresh JKF6 in killing assays. The protective effect of CEACAM1 was evident by the enhanced susceptibility of DI #2 cells, as compared to the Control-RNA cells, to elimination by JKF6 (Fig. 7d). There was no significant difference between melanoma cells that were incubated alone and melanoma cells that survived incubation with inert TIL01-CN cells (Fig. 7d). Similar results were gathered daily throughout the long coincubation experiment (data not shown). Remarkably, Control-RNA cells that survived JKF6 gradually became more resistant to killing by fresh JKF6 cells (Fig. 7e).
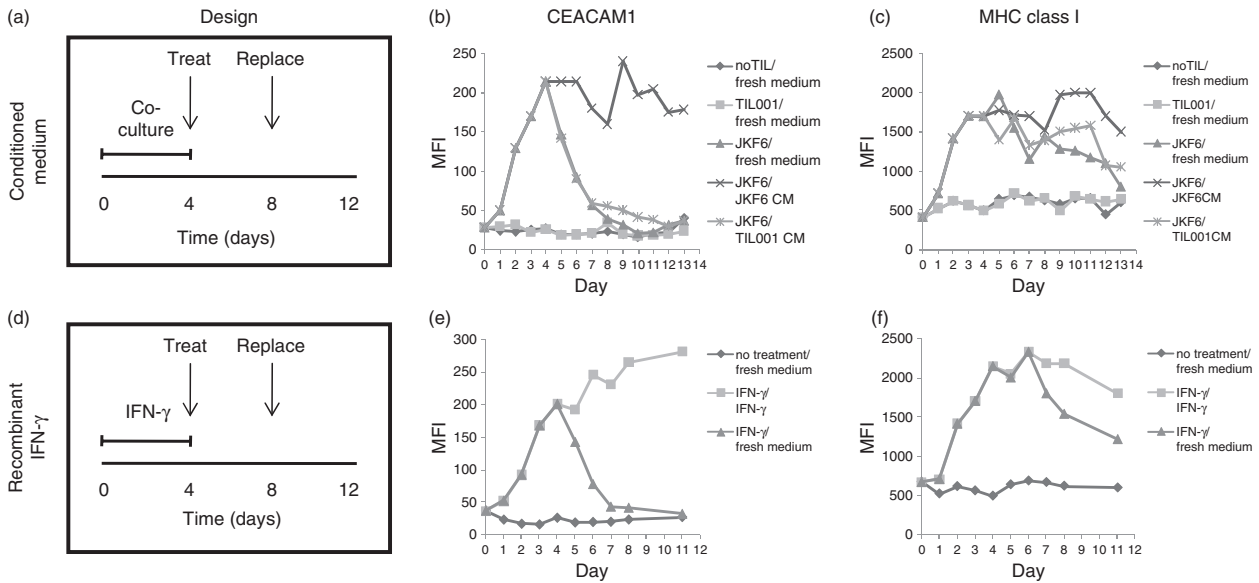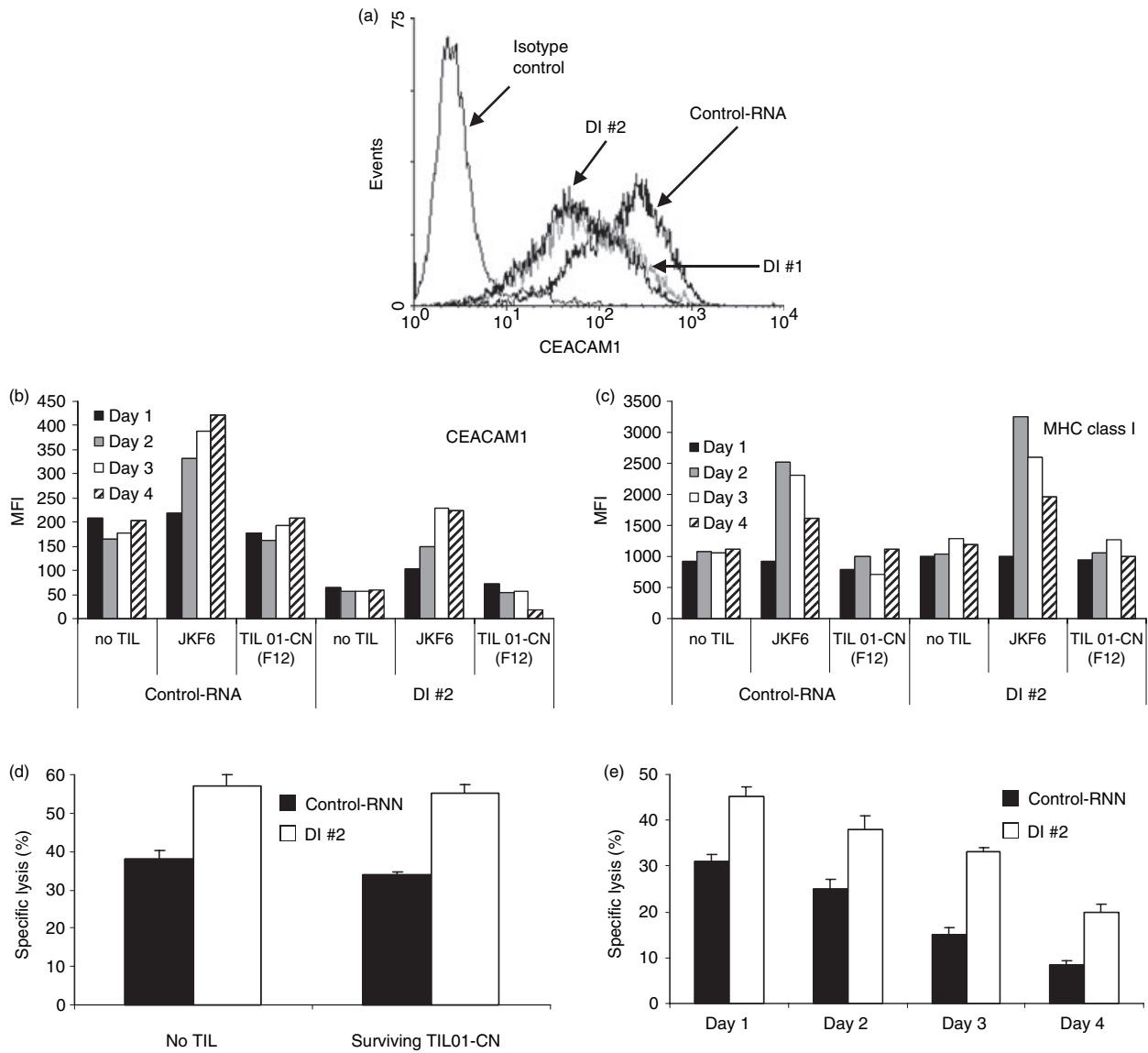
Figure 6. Enhanced CEACAM1 expression on surviving cells is dependent on the continuous presence of interferon-γ (IFN-γ). (a) Experimental design of kinetics after coculture includes: Time (in days); 'Treat' refers to isolation, washing and re-seeding of surviving cells in designated media; 'Replace' refers to replacement of half of the medium with fresh medium of the same kind. (b,c) Expression analyses of CEACAM1 (b) and major histocompatibility coplex (MHC) class I (c) in surviving cells isolated at each day. Each type of treatment includes the conditions of coculture followed by the kind of medium in which surviving cells were re-seeded, as indicated in the legend, and separated by a slanted line. (d) Experimental design of kinetics after incubation with recombinant IFN-γ includes similar components as (a). (e,f) Expression analysis of CEACAM1 (e) and MHC class I (f) in cells isolated at each day. Composition of each treatment is similar to that in (b,c). The figure shows one representative experiment out of three performed.

Therefore, the contribution of CEACAM1 to resistance could not be calculated by simply comparing the susceptibility to fresh TIL of melanoma cells that survive JKF6 to melanoma cells that survive inert TIL01-CN or no TIL. The surviving DI #2 cells displayed similar behaviour, as they also gradually became more resistant (Fig. 7e). Importantly, in all cases the DI #2 cells were more efficiently killed than Control-RNA cells (Fig. 7e, P < 0·01). This shows that the intensity of CEACAM1 expression affects tumour protection. The resistance to killing by fresh JKF6 cells increased over time, as indicated by the Relative Resistance Index (RRI; Table 1). Interestingly, there was no significant difference between Control-RNA and DI #2 cells in the rate at which the RRI increased (Table 1). It seems that in this specific model the feature of tumour cells becoming more resistant during attack occurs at a certain rate. The rate of this process is independent of CEACAM1 initial expression level (Fig. 7a,b, Table 1). Yet, the rate at which CEACAM1 is increased on Control-RNA or DI #2 surviving cells is similar (Fig. 7b), and correlates with the rate of RRI increment. The increasing resistance of surviving cells to fresh TIL may be attributed to several mechanisms on top of increased CEACAM1 expression. Quantification of secreted IFN-γ concentrations was performed concomitantly with the cytotoxicity assays and demonstrated similar results (data not shown). Comparable results were

also obtained with DI #1 cells, which diminished the possibility of a non-specific epiphenomenon (data not shown). These results clearly indicate that tumour cells actively become more resistant during specific attack by reactive lymphocytes. This may be attributed to a combination of putative mechanisms, including CEACAM1 expression level, that directly affect resistance.

Discussion

Tumour transformation occurs continuously *in vivo* so hosts are equipped with various monitoring mechanisms, including the immune system. Normally, immunosurveillance rapidly leads to elimination of the transformed cells, but once tumour masses form, the interrelationship with the immune system changes substantially. The interactions become prolonged and tumours can impose local immunosuppressive conditions,[32,33] effectively evade immunosurveillance[34–38] and even launch counterattacks on tumour-infiltrating lymphocytes.[32,33,39,40] Yamshchikov et al.[41] demonstrated 'immune editing' by the tumour and the consequent adaptation of the dominant immune response *in vivo* in a long-term survivor of melanoma. Nevertheless, immunotherapy still holds great promise for melanoma therapy. It seems that a new generation of cell-based therapies may encompass the cutting edge of future immunotherapy. However, relapse is not uncommon even

Figure 7. Increased CEACAM1 expression contributes to enhanced resistance. Stable transfection of 526mel cells with small interfering RNA for CEACAM1 (DI #1 and DI #2) or scrambled (Control-RNA) were generated as described in the *Materials and methods* section. (a) Relative CEACAM1 expression on Control-RNA cells, DI #1 and DI #2 cells as compared to isotype control staining; (b,c) prolonged coincubation of melanoma cell lines Control-RNA or DI #2 was performed either alone or with reactive JKF6 or inert TIL01 cells. Surviving cells were analysed for the expression of CEACAM1 (b) or major histocompatibility complex (MHC) class I (c) over time. The *y*-axis shows the mean fluorescence intensity (MFI) of each staining; (d–e) Control-RNA and DI #2 melanoma cell lines surviving either TIL01-CN or no tumour-infiltrating lymphocytes (TIL) (d) or JKF6 (e) were isolated and tested in cytotoxicity assays against fresh JKF6 cells; (d) shows the results only for day 1. Error bars represent deviation within the experiment. Differences were statistically significant in all tests with $P < 0.01$ between the killing of Control-RNA and DI #2. The figure shows one representative experiment out of five performed.

following impressive shrinkage in tumour load after TIL treatment. Indeed, one of our patients (05-KG) (whose TIL and cognate melanoma cells were used in this work), exhibited just such a dramatic course, as massive lysis of all evident tumour (lung, mediastinal lymph nodes, liver and spleen) was observed following treatment with TIL, to remarkably achieve complete response. Unfortunately, however, 2 months afterwards the disease recurred with an aggressive variant and the patient died despite maxi-

mal treatment within an additional month. Many reasons may account for this event, such as immune selection or tumour cell adaptation.

In recent years we have discovered and described an inhibitory mechanism of effector functions of NK cells[5,16,17] and TIL[23] via CEACAM1. Further, we have shown that clinical preparation protocols of TIL for ACT result in the up-regulation of CEACAM1 on the surface of transferred TIL over time.[23] The interactions between

Table 1. Increasing resistance of surviving cells to attack by fresh JKF6 cells

	526mel/Control-RNA		526mel/DI #2	
Day	RRI JKF6	RRI TIL01	RRI JKF6	RRI TIL01
1	0·24	0·00	0·24	0·02
2	0·32	0·0	0·31	0·04
3	0·53	0·06	0·45	0·00
4	0·78	0·11	0·65	0·04

Table presents the Relative Resistance Index (RRI) of 526mel cells stably transfected either with scrambled sequence (Control-RNA) or with small interfering RNA to CEACAM1 (DI #2). Melanoma cells that survived prolonged incubation either with TIL01 or with JKF6 were further tested at each time-point in a cytotoxicity assay against fresh JKF6 cells. RRI represents the relative resistance to killing of melanoma cells surviving tumour-infiltrating lymphocytes (TIL) compared to normal melanoma cells and was calculated as follows: 1 − [(fresh JKF6 killing rate of melanoma cells precultured with JKF6 or with TIL01)/(fresh JKF6 killing rate of melanoma cells cultured without any TIL)]. The increase in RRI of melanoma cells in the presence of JKF6 was statistically significant every day as compared to the day before ($P < 0.05$). There was no statistically significant difference in parallel RRI between 526mel/Control-RNA and 526mel/DI #2 cells ($P = 0.17$). The table shows one representative experiment out of three performed.

TIL and melanoma cells *in vivo* are prolonged so we have hypothesized that melanoma cells may actively manipulate CEACAM1 expression to evade ongoing TIL-mediated elimination. We developed an *in vitro* simulation of prolonged interactions by coincubating TIL with melanoma cells for several days and focused on the surviving melanoma cells. Comprehensive experimentation shows that CEACAM1 is gradually up-regulated on melanoma cells that survive *in vitro* attack by reactive lymphocytes. This effect is directly related to the attack by the TIL, because prolonged coincubation of melanoma cells with inert mismatched TIL failed to induce a similar effect. The effect was observed with established TIL clones or with patient-derived primary bulk TIL cultures, either autologous or allogeneic (partially matched HLA haplotype and antigens), as well as with established melanoma cells lines and primary cultures. Although a selective process cannot be entirely excluded, we have positively identified IFN-γ derived from the attacking immune cells as a causative factor for CEACAM1 up-regulation on surviving melanoma cells. Furthermore, when cytotoxic TIL that lack IFN-γ secretion capability were used, we failed to select for a melanoma subpopulation that expresses higher CEACAM1 levels (Fig. 2).

Interferon-γ is an effector protein of the immune system that is secreted chiefly by NK cells, T helper type 1 cells and cytotoxic T lymphocytes.[42] It exhibits pleiotropic effects and alters the expression of multiple gene families.[30] Secretion of IFN-γ is believed to create an anti-tumour milieu because it inhibits the growth of many tumour cell types,[31] induces proapoptotic death signals,[43] hinders angiogenesis[44] and polarizes immune response towards a T helper type 1 response.[45] In this study we demonstrate that surviving melanoma cells couple the response to IFN-γ, an offensive immune agent, with active up-regulation of a tumour-defensive mechanism to become more resistant to further attack by lymphocytes. These results are in accordance with previous works regarding regulation of CEACAM1 expression by IFN-γ.[25,27] Harnessing of host-derived agents by tumours is observed for example in extracellular matrix remodelling and angiogenesis, where the secreted factors are common to both cancer cells and TIL.[46] It should be noted that CEACAM1 expression could not be induced on CEACAM1-negative melanoma by IFN-γ (data not shown). The enhanced CEACAM1 levels closely depend on the continuous presence of IFN-γ, as opposed to the more stable enhanced expression of MHC class I, even after IFN-γ has been removed (Fig. 6). Indeed, following elimination of IFN-γ, CEACAM1 expression dramatically falls by approximately 50% approximately every 24 hr. It has been reported by others that IFN-γ regulates CEACAM1 at the transcriptional and posttranscriptional levels.[25,27]

In conclusion, we demonstrate that melanoma can transiently acquire an enhanced CEACAM1 phenotype, as long as IFN-γ is present. This may allow melanoma cells to 'sense' that they are under lymphocyte-mediated attack, and actively respond accordingly. On the other hand, CEACAM1 may potentially transduce signals into the melanoma cells with a yet to be determined cellular effect. Indeed, it has been reported that CEACAM1 may exert some antiproliferative properties in other cell systems.[6,7] Therefore, the close dependence on the presence of IFN-γ limits CEACAM1 enhancement to the relevant time of attack only.

The TIL did not up-regulate CEACAM1 when exposed to IFN-γ, either during prolonged coincubation or when subjected to the recombinant protein. Indeed, it has been shown that naïve T cells, which are highly responsive to IFN-γ, down-regulate IFN-γ receptor chain 2 when they become effector cells following *in vivo* activation and antigen-driven expansion.[47] Effector cells are submerged in locally high concentrations of IFN-γ so this may be a mechanism by which immune cells avoid the cell-cycle-arresting properties of IFN-γ. Remarkably, the hyporesponsiveness to IFN-γ concomitantly prevents unnecessary up-regulation of CEACAM1 on effector cells, which would have increased the inhibitory effect.

Interestingly, we have observed that the surviving cells become more resistant despite a more prominent up-regulation in MHC class I expression ratio than in CEACAM1 expression ratio. Several explanations may account for this observation. First, HLA allele expression may be differentially affected by IFN-γ, which may result in a

more prominent increase in HLA alleles that do not present immunodominant peptides, such as HLA-C, HLA-E and HLA-G. Alternatively, the MHC class I complex is the target for the TCR, but is concomitantly the target for inhibitory receptors of the killer immunoglobulin-like receptor family (KIRs),[48] which are also expressed by some T cells.[48] Indeed, we have observed some TIL subpopulations that express LIR1, CD94, KIR2DL1 and KIR2DL2 (unpublished results). This may cause an increased flux of inhibitory signals, especially if HLA expression is differentially regulated by IFN-γ. A third explanation would be a shift from the standard proteasome to immunoproteasome, which may change the pool of peptides presented by MHC class I molecules. The induction of tolerogenic pathways such as indoleamine 2,3-dioxygenase (IDO);[49] and additional unidentified mechanisms may also be involved in the emergence of resistant variants, such as down-regulation of certain tumour antigens, adhesion molecules etc. These mechanisms may be either driven by random selection or by specific mechanisms, such as other IFN-γ-sensitive genes. Nevertheless, a protective contribution for enhanced CEACAM1 was still evident, which may exist in parallel with other inhibitory and stimulating alterations caused by IFN-γ (Fig. 7). The added protective effect of enhanced CEACAM1 described here is moderate, probably because the CEACAM1 was only partially silenced (50%, see Fig. 7). The inhibitory signal is thought to be delivered into the attacking lymphocytes following homophilic CEACAM1 interactions through the recruitment of SHP-1 phosphatase to the ITIM sequences[15] of the long cytosolic tail.[11]

In summary, in this work we provide mechanistic evidence that CEACAM1 is actively up-regulated in response to IFN-γ on melanoma cells surviving specific lymphocyte-mediated attack to emerge with transiently enhanced resistance. This dynamic evasion mechanism reveals a potential sophisticated tumour strategy that utilizes an offensive agent as a sensor for the presence of nearby immune attack to actively increase the protection of surviving cells. Recurrence of disease following successful immunotherapeutic treatments, as was observed with patient 05-KG, may be at least partially explained by such a mechanism. The *in vitro* simulation, however, is only a simplistic approximation of a natural process with substantial complexity. Full delineation of the *in vivo* relevance of this mechanism to disease recurrence for instance, requires further extensive investigation. Several approaches and immunological tools can be employed, such as the murine experimental B16 melanoma model and xenotransplantation of human CEACAM1-positive melanoma and tumour-reactive lymphocytes in immune-compromised mice. Both of them allow *in vivo* modelling of tumour–immune cell interactions following establishment of subcutaneous or metastatic melanoma masses

and transfer of appropriate lymphocytes. A main advantage for the use of B16 would be the availability of IFN-γ knockout mice that can serve either as hosts or as donors for adoptively transferred lymphocytes. However, it remains to be determined whether B16 expresses CEACAM1 and whether it is similarly regulated by IFN-γ. It has already been demonstrated that CEACAM1 inhibits T-cell function in the mouse.[15] Vital *in vivo* imaging technologies, such as the cooled charged-coupled device (CCCD) camera, may be capable of illustrating the spatiotemporal characteristics of this process. A cellular reporter system that reflects CEACAM1 expression intensity in live cells, such as a fusion construct of CEACAM1 promoter and *firefly luciferase* can be employed. The contribution of this mechanism to enhanced resistance and recurrence of disease can be directly assessed by elimination of the interferon regulatory factor 1 (IRF1)-responsive element in the CEACAM1 promoter in the transplanted melanoma cells. These cells will express CEACAM1, but will be unable to further modulate CEACAM1 expression. In addition, CEACAM1 knockout mice[50] and a conditional CEACAM1 deletion model[15] have been lately generated by other groups. Utilization of these powerful tools will considerably advance the mechanistic understanding of CEACAM1 involvement in tumour immunobiology. Finally, these findings point to the important role of CEACAM1 in the development of aggressive melanoma and mark it as an attractive target for novel immunotherapy.

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